

AD _____

Award Number: W81XWH-10-1-1009

TITLE: Molecular Profiles for Lung Cancer Pathogenesis and Detection in US Veterans

PRINCIPAL INVESTIGATOR: Pierre Massion, M.D.

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, TN 37240-7830

Á

REPORT DATE: October 201G

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188		
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE October 201G		2. REPORT TYPE Annual		3. DATES COVERED 20September 201F – 19 September 201G	
4. TITLE AND SUBTITLE Molecular Profiles for Lung Cancer Pathogenesis and Detection in US Veterans			5a. CONTRACT NUMBER		
			5b. GRANT NUMBER W81XWH-10-1-1009		
			5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Pierre Massion, M.D. E-Mail: pierre.massion@vanderbilt.edu			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Vanderbilt University Medical Center Nashville, TN 37240-7830			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Á Ö`!q * Á`!Á^& } aÁ^æÁ Á^•^æ&ÖÁ ^ÁÖæ^Á ^&c^aÁ] æ@ æÁæ] ^•Á@[* @`ó•óÁ•] ææ ^Á[{ Á { [^!•Á æóÁ aÁ æó`ó } * Áæ &!Á•á * Á { { [] ÁUU•Á&[••ÁÁ Á æóÁ æ } Á•æ`ó } •Éæ áÁ ^ÁÖæ^Á æææ áÁ @ ^•^}[{ ^Á *^•^Éç ^••á } Á [-æá * Á Á•^Áæ] ^•Á•á * Á[çÁU•Ö•^ Áæ áÁ æ[æ!æ•ÉY ^Áæ[Á•^áÁ } á`^Áæ] æóÁ Á [-áÁ &^Á[] ææ } •Á[{ Á•Á[{ æææ æÉ ^ { æá } æó•á } •Áæ áÁ { [•Áæ áÁ ^!^Áæ ^Á Áæææ Á•^Á^} •ÉY ^ÁÖæ^Á Á•ææá @áÁ ç[{ æÁ ^çáÁ~ ááÁ ÁæææÁ`!Áæ áææ•Á Á![] &ÖÁ] ^&á ^} •Á`!q * Á^æ•ÁÁæ áÁ Á Á•Á æáÁ Ö[çÁ•Á] ææÁ æ] á * Áæ áÁ•Á ^ { æá } æó•^Áç áá•Áæ^Áç ^&c^aÁ Áá áææ æÁæ { æ ^!•Á ^ } * Áæ &!Á Á^Á ç•çáÁ Áç ^Áæ •Á Á•Á b&ÖÁ Á Á Á Á					
15. SUBJECT TERMS Lung Cancer					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	Á¶	19b. TELEPHONE NUMBER (include area code)

Table of Contents

Introduction.....	4
Progress Report (Body).....	
<i>Specific Aim 1.....</i>	<i>5</i>
<i>Specific Aim 2.....</i>	<i>8</i>
<i>Specific Aim 3.....</i>	<i>13</i>
Key Research Accomplishments.....	13
Reportable Outcomes.....	13
Conclusion.....	13
References.....	14

INTRODUCTION

Lung cancer continues to be the leading cause of cancer-related death in both men and women in the United States ¹. The majority of lung cancers are non-small cell lung cancers (NSCLCs) that include squamous cell carcinomas (SCCs) and adenocarcinomas ². Lung cancer mortality is high in part because most cancers are diagnosed after regional or distant spread of the disease had already occurred and due to the lack of reliable biomarkers for early detection and risk assessment ². The identification of new effective early biomarkers will improve clinical management of lung cancer and is linked to better understanding of the molecular events associated with the development and progression of the disease.

It has been suggested that histologically normal-appearing tissue adjacent to neoplastic lesions display molecular abnormalities some of which are in common with those in the tumors ³. This phenomenon, termed field of cancerization, was later shown to be evident in various epithelial cell malignancies, including lung cancer ^{4,5}. Loss of heterozygosity (LOH) events are frequent in cells obtained from bronchial brushings of normal and abnormal lungs from patients undergoing diagnostic bronchoscopy and were detected in cells from the ipsilateral and contralateral lungs ⁶. More recently, global mRNA expression profiles have been described in the normal-appearing bronchial epithelium of healthy smokers ⁷. In addition, modulation of global gene expression in the normal epithelium in health smokers is similar in the large and small airways and the smoking-induced alterations are mirrored in the epithelia of the mainstem bronchus, buccal and nasal cavities ⁸. Finally, our group has previously shown that gene-expression profiles in cytologically normal mainstem bronchus epithelium can distinguish smokers with and without lung cancer and can serve as an early diagnostic biomarker for lung cancer⁹.

In this program, in Specific Aim 1, we will extend our work in this field by spatially mapping the molecular field of injury associated with smoking-related lung cancer. In smokers undergoing resection of lung lesions, high-throughput mRNA expression analyses are being performed on cytological specimens (brushings) obtained at intraoperative bronchoscopy from the nasal epithelium, main carina and ipsilateral and contralateral proximal and distal bronchi (relative to the location of the resected lung lesion), as well as on specimens obtained at lobectomy from sub-segmental bronchus (adjacent to tumor) and from the resected NSCLC tumors. Towards this aim, we are comparing and contrasting global gene expression patterns across all the specimens from the entire field and corresponding NSCLC tumors. We are currently performing RNA-sequencing and microarray profiling of nasal epithelia, airway epithelial cells collected from both bronchoscopy and lobectomy specimens as well as of corresponding tumors (NSCLC patients) or benign lesions (cancer-free individuals).

In Specific Aim 2, we are using laser capture microdissection to obtain specific cell populations (basal cells or type II alveolar cells, depending on the NSCLC histology/location) as well as premalignant lesions and epithelial components of the tumors. These cell populations are being profiled with RNA-seq to determine their gene expression signatures to increase our understanding of premalignancy. We are analyzing the gene expression profiles that are associated with progression from a benign cell population to premalignancy and with progression from a benign cell population to true malignancy.

In future studies, in Specific Aim 3, we will use expression signatures and biomarkers derived from the results of aims 1 and 2 to develop and test airway-based biomarkers capable of diagnosing lung cancer in current or former smokers using minimally invasive sites. This report details the progress made during the second year of research.

Molecular Profiles for Lung Cancer Pathogenesis and Detection in U.S. Veterans

Specific Aim 1: **To increase our understanding of the molecular basis of the pathogenesis of lung cancer in the “field cancerization” that develops in current and former smokers.**

Summary of Research Findings

A. Collection of airway epithelial samples from both bronchoscopy and lobectomy specimens from smokers with and without lung cancer (Sub-specific Aims 1A and 1C):

We have recruited 35 study participants undergoing resection of lung tumor or benign lung lesions to collect tissue samples for the studies in Aim 1. From these subjects who were recruited at all 4 participating institutions, we have collected nasal epithelium, proximal and distal bronchial airway epithelium obtained at bronchoscopy (ipsilateral and contralateral to the tumor) as well as the tumor/benign lesion, adjacent normal parenchyma, and subsegmental bronchial epithelium at time of lobectomy. A summary of subjects recruited at all 4 sites is provided in Table 1 and their demographics are shown in Table 2.

The samples are currently being analyzed by both next generation RNA-sequencing (RNA-Seq) using the Illumina HiSeq 2000 platform and microarray profiling using the Human Gene 2.0 ST platform from Affymetrix. RNA-Seq and microarray analysis are being performed at BU and MD Anderson Cancer Center, respectively. Total RNA from all samples have been isolated using the miRNeasy kit from Qiagen. RNA sequencing will facilitate the discovery of novel transcripts in the molecular field of injury as well quantifying expression of those that cannot be characterized by microarray technology. This study, for the first time, will allow us to 1) perform next generation sequencing in addition to microarray profiling analysis of the molecular field of injury in the airway; 2) study samples obtained from four different institutions in the nation using common SOPs and 3) characterize the complete topological map of the molecular field of injury/cancerization between both NSCLC patients and cancer-free individuals. We anticipate that RNA-Seq and microarray profiling will be completed by the end of the year with subsequent bioinformatic and functional analysis along with validation of expression studies completed by Spring 2013. We anticipate that expression profiles in the NSCLC molecular field of injury will harbor transcripts, both novel and established, that may exhibit potential for use as airway biomarkers that can be developed and tested for lung cancer detection using minimally invasive sites in Specific Aim 3 of this award.

Table 1. Molecular mapping of the field of injury in NSCLC and cancer-free patients

Institution	RNA-Seq (cases)			Microarray (cases)		
	ADC	SCC	No Cancer	ADC	SCC	No Cancer
MD Anderson	4	2	0	4	3	0
BU	2	1	3	0	2	4
UCLA	2	2	1	3	2	2
Vanderbilt	1	1	1	4	3	1
Number	9	6	5	11	10	7
Total Nb. of cases analyzed	20			28		
Total Nb. of samples analyzed	156			183		

RNA-Seq, RNA sequencing; ADC, adenocarcinoma; SCC, squamous cell carcinoma; BU, Boston University; UCLA, University of California Los Angeles.

Table 2. Demographics of study participants

Ethnicity	Male	Female
White	14	5
Black	7	3
Hispanic	1	0
Asian	2	1
American Indian	0	0
Other	1	0
Unknown	0	0

Tissue collection:

The collection protocol SOP put in place. The Table 3 shows the samples collected at Vanderbilt University including tumor, brushings from large and small airways and areas of normal lung. With the collaboration between Drs Massion (PI) and Dr. Eisenberg (Pathologist), the process is in place and we anticipate enrolling at least 20 patients per year to this protocol.

Table 3. 10 patients collected and samples shipped to BU for RNA extraction and sequencing analysis.

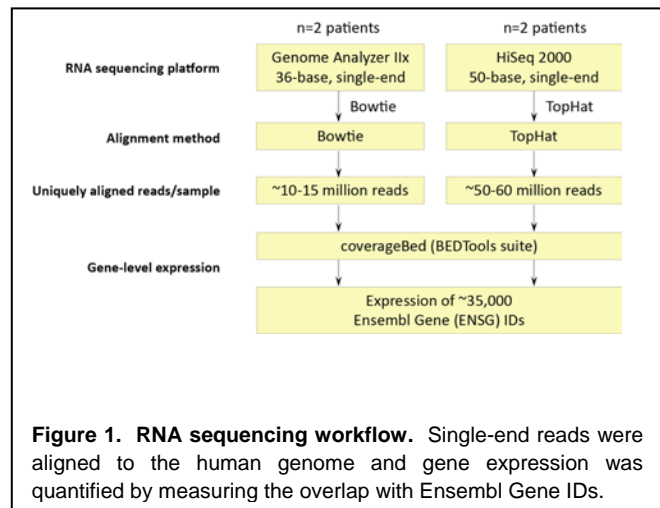
	Patient ID	LCB ID	Sample Type	Fixative	Sample ID	Storage Temperature in Centigrade	Location of Brushes
1	8841	2012-4-1-800-1	Normal Tissue	RNA Later	534048	-80	Tumor is central - LUL
			Normal Tissue		534049	-80	B1 closest to tumor
			Tumor Tissue	RNA Later	534050	-80	B2 same bronchus as B1 - peripheral
			Tumor Tissue		534051	-80	B3 and B4 - different airway -
			Brushes B1-B4	Qiazol	534052-55	-80	
2	8836	2012-4-1-802-1	Normal Tissue	RNA Later	534293	4	Tumor is peripheral (s/p chemorad) - RUL
			Normal Tissue		534294	-80	B3 closest to tumor
			Tumor Tissue	RNA Later	534295	4	B2 same airway as B3 - more proximal
			Tumor Tissue		534296	-80	B1 different airway - proximal
			Brushes B1-B3	Qiazol	534299-301	-80	scant tumor left on specimen after chemorad
3	8836	2012-4-1-803-1	Normal Tissue	RNA Later	534326	4	Tumor is central - LUL
			Normal Tissue		534327	-80	B1 closest to tumor
			Tumor Tissue	RNA Later	534329	4	B2 same airway as B1 - distal
			Tumor Tissue		534328	-80	B3 different airway
			Brushes B1-B3	Qiazol	534330-32	-80	
4	9002	2012-5-1-811-1	Normal Tissue	RNA Later	535568	4	Tumor is peripheral - RLL
			Normal Tissue		535569	-80	B4 closest to tumor
			Tumor Tissue	RNA Later	535570	4	B1 and B2 same airway as B4 - more proximal
			Tumor Tissue		535571	-80	B3 different airway - proximal
			Brushes B1-B4	Qiazol	535572-75	-80	
5	9006	2012-5-1-814-1	Normal Tissue	RNA Later	535692	4	Tumor is central - LUL
			Normal Tissue		535690	-80	B1 closest to tumor
			Tumor Tissue	RNA Later	535693	4	B2 and B4 same airway as B1 - distal
			Tumor Tissue		535691	-80	B3 different airway
			Brushes B1-B4	Qiazol	535694-97	-80	
6	9047	2012-6-1-821-1	Normal Tissue	RNA Later	536361	4	Tumor is Central - LUL
			Normal Tissue		536360	-80	B1 is closest to tumor
			Tumor Tissue	RNA Later	536363	4	B2 on a different airway than B1 distal.
			Tumor Tissue		536362	-80	B3 on an opposite airway distal.
			Brushes B1-B3	Qiazol	536364-66	-80	
7	9078	2012-6-1-824-1	Normal Tissue	RNA Later	536782	4	Tumor is peripheral - LLL
			Normal Tissue		536781	-80	B1 is distal to tumor
			Tumor Tissue	RNA Later	536784	4	B2 is closest to tumor on same airway as B1.
			Tumor Tissue		536783	-80	B3 is on a different airway distal.
			Brushes B1-B3	Qiazol	536785-87	-80	
8	9138	2012-7-1-828-1	Normal Tissue	RNA Later	537331	4	Tumor is peripheral - LUL
			Normal Tissue		537330	-80	B1 is closest to tumor.
			Tumor Tissue	RNA Later	537329	4	B2 on same airway as B1 distal.
			Tumor Tissue		537328	-80	B3 is on a different airway distal.
			Brushes B1-B3	Qiazol	537332-34	-80	
9	9258	2012-8-1-840-1	Normal Tissue	RNA Later	538648	4	Tumor is perripheral - LLL
			Normal Tissue		538649	-80	B2 is closest to tumor on the same airway as B1.
			Tumor Tissue	RNA Later	538646	4	B3 and B4 are distal to tumor on different airway.
			Tumor Tissue		538647	-80	
			Brushes B1-B4	Qiazol	538650-653	-80	
10	9401	2012-9-1-848-1	Normal Tissue	RNA Later	539677	4	Tumor is peripheral - RUL
			Normal Tissue		539678	-80	B1 is proximal to tumor on same airway as B2.
			Tumor Tissue	RNA Later	539675	4	B2 is distal to tumor.
			Tumor Tissue		539676	-80	B3 is distal to tumor on different airway.
			Brushes B1-B3	Qiazol	539679-681	-80	

Specific Aim 2: To increase our understanding of the role of tumor-initiating stem/progenitor cells in the pathogenesis of lung cancer in the “field cancerization” that develops in current and former smokers.

Summary of Research Findings:

A. Feasibility of sequencing small amounts of RNA from laser captured samples that reflect different pathologic stages of lung carcinogenesis (Sub-specific Aim 2B):

Specific regions of normal basal cells, premalignant metaplastic/dysplastic cells, and squamous carcinoma cells were successfully selected by laser microdissection. This was described and detailed in the previous annual report (Year 1). Adequate amounts of RNA were isolated from these cells for library preparation and high throughput sequencing (RNA-seq), and decent quantities of libraries with appropriate size ranges were generated. This was described and detailed in the previous annual report (Year 1). Samples were sequenced on Illumina Genome Analyzer IIx or HiSeq 2000 instruments, producing single-end reads with quality control Phred scores above 30.



Sequence alignment and quantification of gene expression

The reads produced by sequencing each RNA sample were aligned to the human genome (build hg19). Uniquely aligned reads were then used to compute gene expression estimates by measuring the coverage of each of ~35,000 Ensembl Gene loci using the coverageBed utility in the BEDTools suite. This workflow is illustrated in **Figure 1**.

Identification of genes with progression-associated expression patterns

To identify genes whose expression was associated with progression from normal to intermediate (metaplastic or dysplastic) to tumor cells, a three-step procedure was used in Dr. Spira's lab. First, genes with low expression (median expression was below 100 reads), which are more likely to be false positives, were removed from analysis. Next, to simplify the analysis, a concordance filter was then applied in order to consider only those genes whose expression changed in the same direction in both intermediate and tumor cells relative to normal cells.

B. Identification of additional archived clinical specimens for laser microdissection of tumor-initiating stem/progenitor cells:

Additional archived clinical specimens from which we previously extracted DNA and RNA from rare target cells using laser microdissection were identified. Within the same individual, the following regions/cells were present: normal epithelium, normal basal cells, dysplastic cells in preinvasive tissue, as well as their respective basal cells, and invasive tumor cells. The cases identified included biopsy specimens and resected tumors, and each was confirmed to be amenable for microdissection after review of freshly cut H&E stained slides by our collaborating

pathologists. Using archived clinical specimens available in biorepositories at each site, we have sufficient material to complete the studies described in sub-specific Aim 2B.

In collaboration with Dr. Gomperts, we provided a series of preinvasive lesions. Our knowledge about the field of cancerization is limited and understanding the molecular determinants of tumor development is critical to the area of research. Our collaboration with Dr. Gomperts laboratory is to compare molecular profiles of tumor-initiating stem/progenitor cells from normal airway epithelium, preinvasive lesions and invasive lung tumor tissues and evaluate the role of airway epithelium tumor-initiating stem/progenitor cells in lung cancer pathogenesis in current and former smokers. The hypothesis is that the airways of lung cancer patients have greater population of cells with stem/progenitor-like characteristics, population of cells that we could find in selected individuals at high risk of developing lung cancer. Selection and molecular characterization of this subpopulation may lead to the identification of candidate biomarkers that are important for understanding early events of lung cancer pathogenesis. This may bring relevance to identifying persons at highest risk of developing lung cancer and potentially developing this knowledge into new therapeutic targets.

We specifically provided from our archived materials, tissue specimens we selected from which we could extract, using laser capture micro-dissection, DNA and RNA from normal epithelium, normal basal cells, dysplastic cells in preinvasive tissue as well as their respective basal cells, and compare those to invasive tumor cells from the same individuals. From the inventory of the VUMC biorepository we were able to find biopsy specimens and resected tumor tissues that were amenable for micro-dissection. Dr. Eisenberg, pathologist in our group at VUMC and the Gomperts laboratory has reviewed each H&E stained slide. The specifics of the specimens sent to the Gomperts laboratory included slides and tissue blocks of three biopsies and one resected tumor as described in Table 4.

Table 4. Samples sharing with Dr. Gomperts.

Date of shipment	ID	Description	Unstained slides	H&E slides	Tissue blocks
6/15/2010	2006-9-1-19-16	Biopsy, Main stem	16	1	
	2007-3-1-435-9	Biopsy, Left main stem lesion	16	1	
	2007-4-5-466-9	Biopsy, Lesion	16	1	
	2005-8-1-240-1	Resected tumor	16	1	
1/25/2011	2006-9-1-19-16	Biopsy, Main stem			1
	2007-3-1-435-9	Biopsy, Left main stem lesion			1
	2007-4-5-466-9	Biopsy, Lesion			1
	2005-8-1-240-1	Resected tumor			1
5/10/2012	S05-23133, 9A	Resected tumor	2	1	
	S05-23133, 9E	Resected tumor	2	1	
	S05-28605, 7A	Resected tumor	2	1	
	S05-28605, 7B	Resected tumor	2	1	
	S09-11290, 2C	Resected tumor	2	1	
	S09-11290, 8A	Resected tumor	2	1	
	S09-11290, 8I	Resected tumor	2	1	
	S09-11290, 8K	Resected tumor	2	1	
6/25/2012	S05-23133, 9A	Resected tumor	10 (2 sections/slide)		
	S05-23133, 9E	Resected tumor	20 (1 sections/slide)		

C. Proteomic Studies

As previously described, the UCLA group identified a molecular profile dominated by the Snail transcription factor that appears to drive epithelial mesenchymal transition (EMT) and tumor-initiating characteristics in the airway epithelium, as modeled *in vitro* and *in vivo*. Snail is also over-expressed in human bronchial epithelial cells in premalignant lesions *in situ* concomitant with markers of EMT and stemness. To validate the technology for analyzing tumor-initiating stem cells from *in situ* specimens, we performed preliminary *in vitro* experiments to assess the impact of this transcription factor on protein expression profiles of human bronchial epithelial cells. In this context, we performed Shotgun Proteomic Analysis comparing human bronchial epithelial cells and the same cells ectopically over-expressing Snail.

Cell pellets were collected at UCLA and prepared for LC-MS/MS shotgun proteomics and analyzed in the Jim Ayers Institute at Vanderbilt, as described in our year 1 progress report. Briefly, each 0.2 mg protein aliquot was digested and resolved by isoelectric focusing into 15 fractions that were subsequently analyzed by LC-MS/MS. Thus, there were 6 measurements (2 technical replicates for 3 samples) for the control group and 6 for the Snail+ group. Raw MS/MS data were evaluated using MyriMatch and IDPicker software. Differentially expressed proteins were then identified using Quasi-Tel pair wise comparison.

The initial dataset was robust, with 2809 protein groups identified overall; a protein group usually represents a single protein, but it is occasionally a small group of indistinguishable proteins with identical peptides. The overall numbers of protein groups in the control and Snail+ bronchial epithelial cells were similar (2229 and 2738, respectively). The following general observations were made: (1) Known markers of EMT were over-expressed in the Snail+ cells. (2) Other structural/motility proteins consistent with an EMT phenotype were also over-expressed in the Snail+ cells.

To augment our ability to identify proteins relevant to the molecular pathogenesis of lung cancer across the broadest possible patient population, we will perform shotgun proteomics on additional samples. A panel of bronchial epithelial cells isolated from patients and engineered to over-express Snail have been plated in western blot and anchorage independent growth (AIG) assays. Via these assays, we will re-confirm their Snail expression and Snail-driven malignant conversion prior to their proteomic evaluation. Each of the cell types in this panel has previously demonstrated numerous Snail-driven cancer-associated phenotypes, including EMT, stemness, AIG, and/or tumor growth and metastatic behavior in mice. At the conclusion of these assays, cells maintained in culture in parallel will be collected, prepared for shotgun proteomics, and analyzed, as previously described. Additionally, tumor-initiating Snail+ALDH+CD44+CD24- bronchial epithelial cells will be subjected to shotgun proteomics in the same manner. This relatively rare cell type will be isolated by fluorescence activated cell sorting (FACS), and the resulting cell pellets will be frozen. Multiple pellets will be pooled to generate material sufficient for evaluation; this again models laser capture microdissection (LCM) isolation of stem/progenitor target cells from normal/SM/SCC and normal/AAH/ADC regions of archived clinical specimens.

By evaluating additional samples, including rare tumor-initiating stem cells, we anticipate arriving at a more robust protein signature relevant to lung carcinogenesis. Models and software developed in the Jim Ayers Institute at Vanderbilt are more appropriately applied to studies with these multiple inputs. The new protein signature that emerges will be further strengthened via Multiple Reaction Monitoring (MRM) performed on the remaining samples by the Vanderbilt group. MRM using mass spectrometry is a highly sensitive and selective method for the

targeted quantitation of protein or peptide abundances in complex samples. While shotgun proteomics detects all protein changes in the sample in an unfocused fashion, MRM is targeted and highly selective, allowing us to specifically look for proteins of interest.

To this end, we have generated a list of candidate proteins for MRM utilizing shotgun proteomic, mRNA array, and miRNA array datasets generated from the same Snail+ cells. Candidates with the greatest fold change and level of significance were included. Candidates at the intersection of each of these lists, as evaluated by Ingenuity Pathway Analysis (IPA), were also included. Finally, additional candidates of interest were included based on our hypothesis-driven studies of lung carcinogenesis, including mediators of inflammation, EMT, stemness, metabolism, apoptosis-resistance, as evaluated in the PIs' lab-based studies over the past several years. While we have already generated this candidate list for MRM, the list will be further refined as we expand our shotgun proteomic analysis to include additional samples.

Finally, during the preceding funding period, we developed a Microsoft Access database with the intent of including an additional parameter, "druggability", in the selection of top candidates for further validation and detailed functional studies. The first iteration of the database was created by integrating the protein, mRNA, and miRNA datasets previously described with a lung cancer-specific terms list (**see Figures**). These were then linked to information regarding proteins/genes/miRNAs for which agents are in use or in the pipeline along with additional clinical utility parameters, such as how successful the agent is and its range of use. This database will be refined as we expand our shotgun proteomic analysis to additional samples and as we receive inputs from the sequencing and array studies in the other aims. This database will serve as an important new tool for selecting the best protein candidates to include in our upcoming MRM studies.

A		B		C		D	
List of Proteins		List of Genes		List of Genes		List of Genes	
Shotgun Proteomics / All with Significant P-Values		DAE Analysis / >1.33-Fold Change		DAE Analysis / >1.33-Fold Change		DAE Analysis / >1.33-Fold Change	
Symbol	Protein name	P-Value	Baseline Mean	Experimental Mean	Fold V-S	Fold S-V	Linear scale (2 nd)
AAHS2	ENSG00000124608 AAHS2 alanyl-tRNA synthetase 2, mitochondrial (putative)	0.2453	7409.88	6603.25	1.1222	0.8911	10.96
AC092837.2	ENSG00000124938 AC092837.2 Mesencephalic astrocyte-derived neurotrophic factor 1	0.2453	5896.88	4537.50	1.2996	0.7695	-25.38
ACTN4	ENSG00000139402 ACTN4 actinin, alpha 4	0.2453	7484.38	7109.88	1.0527	0.9500	11.86
AL353997.5	ENSG00000186831 AL353997.5 Putative uncharacterized protein >ENSPO0000319235	0.4142	3530.25	3298.50	1.0703	0.9344	-12.18
ANKA2	ENSG00000182718 ANKA2 annexin A2	0.6985	2306.13	2157.00	1.0691	0.9353	30.68
ATP5B	ENSG00000119555 ATP5B ATP synthase, H+ transporting, mitochondrial F1 complex, b subunit	0.2207	15089.50	16558.00	0.9113	1.0973	-16.08
BCAM	ENSG00000187244 BCAM basal cell adhesion molecule (Lutheran blood group)	0.6985	1272.13	1347.88	0.9438	1.0595	14.81
C13orf72	ENSG00000099812 C13orf72 Uncharacterized protein C13orf72	0.6985	874.63	799.75	1.0936	0.9144	16.69
CAU1	ENSG00000128595 CAU1 calumenin	0.2453	2086.25	1371.63	1.5210	0.6575	30.90
CKAP4	ENSG00000136626 CKAP4 cytoskeleton-associated protein 4	0.6985	874.75	1037.38	0.8432	1.1859	15.32
CLTC	ENSG00000141367 C	1.0000	1259.63	1334.50	0.9439	1.0594	-107.13
DYNC1H1	ENSG00000197102 D	0.2453	789.25	732.75	1.0771	0.9284	-14.69
ECH1	ENSG00000104823 E	0.4142	505.25	576.88	0.8758	1.1418	-14.00
EIF4G1	ENSG00000114867 E	1.0000	257.69	258.00	0.9988	1.0012	17.30
EPNS	ENSG00000113626 E	1.0000	277.50	284.88	0.9741	1.0266	16.50
FAM120A	ENSG00000040828 F	0.2453	425.38	447.00	0.9516	1.0508	10.57
FASN	ENSG00000169710 F	0.4142	229.13	279.13	0.8209	1.2182	-12.06
FDT1	ENSG00000079459 F	1.0000	5335.38	5179.50	1.0301	0.9708	24.35
FKBP10	ENSG00000141756 F	0.6985	294.13	315.13	0.9334	1.0714	19.45
FN1	ENSG00000115414 F	0.6985	766.38	669.50	1.1447	0.8736	-10.67
HNRNP1A1	ENSG00000113486 H	1.0000	2865.75	3190.38	0.8982	1.1133	11.09
HS90B1	ENSG00000166598 H	0.2453	2029.50	1523.38	1.3322	0.7506	11.35
hsa-let-7a	hsa-let-7a	0.2453	4916.00	3253.38	1.5110	0.6618	11.35
hsa-let-7b/mmu-let-7b/miR-10b	hsa-let-7b/mmu-let-7b/miR-10b	0.2453	7409.88	6603.25	1.1222	0.8911	10.96
hsa-let-7c/mmu-let-7c/miR-10b	hsa-let-7c/mmu-let-7c/miR-10b	0.2453	7484.38	7109.88	1.0527	0.9500	11.86
hsa-let-7d/mmu-let-7d/miR-10b	hsa-let-7d/mmu-let-7d/miR-10b	0.4142	3530.25	3298.50	1.0703	0.9344	-12.18
hsa-let-7e/mmu-let-7e/miR-10b	hsa-let-7e/mmu-let-7e/miR-10b	0.6985	2306.13	2157.00	1.0691	0.9353	30.68
hsa-let-7f/mmu-let-7f/miR-10b	hsa-let-7f/mmu-let-7f/miR-10b	0.2207	15089.50	16558.00	0.9113	1.0973	-16.08
hsa-let-7g/mmu-let-7g/miR-10b	hsa-let-7g/mmu-let-7g/miR-10b	0.6985	1272.13	1347.88	0.9438	1.0595	14.81
hsa-let-7i/mmu-let-7i/miR-10b	hsa-let-7i/mmu-let-7i/miR-10b	0.6985	874.63	799.75	1.0936	0.9144	16.69
hsa-miR-100/mmu-miR-100f	hsa-miR-100/mmu-miR-100f	0.2453	2086.25	1371.63	1.5210	0.6575	30.90
hsa-miR-100b/mmu-miR-100f	hsa-miR-100b/mmu-miR-100f	0.6985	874.75	1037.38	0.8432	1.1859	15.32
hsa-miR-100a	hsa-miR-100a	1.0000	1259.63	1334.50	0.9439	1.0594	-107.13
hsa-miR-100b/mmu-miR-100f	hsa-miR-100b/mmu-miR-100f	0.2453	789.25	732.75	1.0771	0.9284	-14.69
hsa-miR-107	hsa-miR-107	0.4142	505.25	576.88	0.8758	1.1418	-14.00
hsa-miR-10a/mmu-miR-10a	hsa-miR-10a/mmu-miR-10a	1.0000	257.69	258.00	0.9988	1.0012	17.30
hsa-miR-10b/mmu-miR-10b	hsa-miR-10b/mmu-miR-10b	1.0000	277.50	284.88	0.9741	1.0266	16.50
hsa-miR-1201	hsa-miR-1201	0.2453	425.38	447.00	0.9516	1.0508	10.57
hsa-miR-1216	hsa-miR-1216	0.4142	229.13	279.13	0.8209	1.2182	-12.06
hsa-miR-1246	hsa-miR-1246	1.0000	5335.38	5179.50	1.0301	0.9708	24.35
hsa-miR-1249	hsa-miR-1249	0.6985	294.13	315.13	0.9334	1.0714	19.45
hsa-miR-1255a	hsa-miR-1255a	0.6985	766.38	669.50	1.1447	0.8736	-10.67
hsa-miR-1259	hsa-miR-1259	1.0000	2865.75	3190.38	0.8982	1.1133	11.09
hsa-miR-125a-5p/mmu-miR-125	hsa-miR-125a-5p/mmu-miR-125	0.2453	2029.50	1523.38	1.3322	0.7506	11.35
hsa-miR-125b/mmu-miR-125	hsa-miR-125b/mmu-miR-125	0.2453	4916.00	3253.38	1.5110	0.6618	11.35

Snail datasets used to build 'druggable' candidate selection Microsoft Access database. Datasets include mRNA array, miRNA array, and shotgun proteomics results.

Army Award W81XWH-10-1-1009; Annual Report from Vanderbilt PI: Massion, Pierre
Annual Report: Reporting Period 20 Sept 2011 – 19 Sept 2012

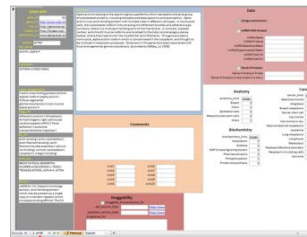
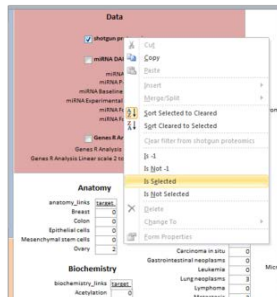
Lung Cancer Terms:

Non-small cell lung cancer (NSCLC)	Telomerase
Atypical adenomatous hyperplasia (AAH)	Anchorage independent growth
Squamous cell carcinoma	Malignant conversion
Squamous metaplasia (SM)	Motility
Premalignancy	Migration
Chronic obstructive pulmonary disease (COPD)	Invasion
Idiopathic pulmonary fibrosis (IPF)	Metastasis
FEV1	MicroRNA
FEV1/FVC ratio	Non-coding RNA
Tiffeneau index	Small Non-coding RNA
Asthma	Transcription factor
Emphysema	Zinc-finger transcription factor
Smoking	Transcriptional repressor
Chronic inflammation	Chemoprevention
Airway destruction	Inflammatory mediators
Epithelial-to-mesenchymal transition (EMT)	Phosphorylation
Plasticity	DNA binding
Tumor initiating stem cells	Tumor suppressor
Progenitor cells	Somatic mutation
Stem cell niche	Loss-of-function
Microenvironment	Alternative splicing
Immune suppression	RNA interference
Immune escape	Protein-protein interactions
Immune surveillance	Tyrosine kinase receptor
Autophagy	G-protein coupled receptor
Cancer-associated fibroblasts	Seven-transmembrane receptor
Apoptosis	Glycolysis
Angiogenesis	Oxidative stress
Senescence	Reactive oxygen species
Contact inhibition	Drug resistance
Immortality	Exosomes
	Circulating microRNA
	Parallel progression model

Lung cancer terms list used to build 'druggable' candidate selection database.

To Select Genes in The "Shotgun Proteomics" data – right click on the dataset

- Then click on is "Selected"
- The shotgun proteomics box is a checkbox. It is a yes no selection.
- This maneuver will select all genes in the original shotgun proteomics Excel spreadsheet.



69 genes

- Before selecting there were 42073 genes to scroll through.
- It is now possible to use the arrows to scroll through just the 69 genes in the proteomics dataset.

Creation of MicroSoft Access database. Selection of datasets to overlay and subsequent narrowing of candidate list.



Entrez Info – Gray
Entrez Summary – Blue
Comments – Orange
Druggability – Red
Data – Pink
Literature Links – White

The data submitted by the Dubinett Lab: mRNA candidates; miRNA candidates; protein candidates.

All fields locked except Comments Section. User can use this section for notes and to put in links to interesting abstracts or web pages.

Creation of MicroSoft Access database. Dataset integration and candidate selection based on "druggability".

Specific Aim 3: Test airway-based mRNA and microRNA biomarkers of diagnosing lung cancer in current and former smokers at high risk for lung cancer in minimally invasive sites.

The studies on this Aim will be carried out in Years 3 and 4 of the grant.

In collaboration with Dr. Liebler, we have developed a series of MRM assays that we can test in the airways of individuals for lung cancer. The methods developments have recently been published in MCP^{9,10}. This validation effort proposed in aim 3 has not formally started because we have not settled on candidates to be tested by MRM.

KEY RESEARCH ACCOMPLISHMENTS

1. Collected matched epithelial cells from the nose, proximal and distal airways and tumor/adjacent normal lung from 35 patients undergoing surgical resection of lung lesions at all four participating institutions.
2. Isolated RNA from all airway samples collected and begun to profile RNA using RNA-Seq (20 cases, 156 samples) and microarray (28 cases, 183 samples) platforms in order to characterize the spatial map of the molecular field of injury in NSCLC patients and cancer-free individuals.
3. Performed RNA sequencing on cell populations in matched sets of histologically normal airway, premalignant lesions and tumors from the same individuals, and identified candidate genes that increase in expression in premalignancy and in tumors. We identified candidates that we are validating by PCR and will validate by MRM analysis.
4. Identified and shared a set of preinvasive lesions matching their pair normal and invasive tumors to compare molecular profiles of tumor-initiating stem/progenitor cells from these groups and evaluate the role of airway epithelium tumor-initiating stem/progenitor cells in lung cancer pathogenesis in current and former smokers.
5. Established MRM assays for candidate biomarkers of early lung cancer.

REPORTABLE OUTCOMES

Abstracts:

Ooi AT, Gower AC, Zhang KX, Vick J, Caballero N, Massion PP, Wistuba II, Walser TC, Dubinett SM, Pellegrini M, Lenburg ME, Spira A and Gomperts BN. Molecular Profiles to Improve our Understanding of Lung Cancer Pathogenesis in U.S. Veterans. NIH Lung Cancer SPORE Meeting. Pittsburgh. July 2012.

CONCLUSIONS

During our second year of research, we have collected epithelial samples throughout the respiratory from smokers with and without lung cancer using common SOPs across all 4 participating institutions, and we have initiated whole-genome gene-expression profiling of these samples using both RNA-seq and microarrays. We also used a unique approach to profile cell populations from the normal airway, premalignant lesions and tumors and were able to validate these genes. We have established proteomics methods required to validate our candidates in bronchial specimens during years 3 and 4 of the award. Both the spatial mapping and the premalignant tissue studies are expected to yield airway biomarkers for lung cancer to be tested in future aims of this project

REFERENCES

1. Jemal A, Bray F, Center MM, et al: Global cancer statistics. *CA Cancer J Clin* 61:69-90, 2011
2. Herbst RS, Heymach JV, Lippman SM: Lung cancer. *N Engl J Med* 359:1367-80, 2008
3. Slaughter D, Southwick H, Smejkal W: Field cancerization in oral stratified squamous epithelium: clinical implications of multicentric origin. *Cancer* 6:963-968, 1953
4. Steiling K, Kadar AY, Bergerat A, et al: Comparison of proteomic and transcriptomic profiles in the bronchial airway epithelium of current and never smokers. *PLoS One* 4:e5043, 2009
5. Wistuba, II, Gazdar AF: Lung cancer preneoplasia. *Annu Rev Pathol* 1:331-48, 2006
6. Powell CA, Klares S, O'Connor G, et al: Loss of heterozygosity in epithelial cells obtained by bronchial brushing: clinical utility in lung cancer. *Clin Cancer Res* 5:2025-34, 1999
7. Spira A, Beane J, Shah V, et al: Effects of cigarette smoke on the human airway epithelial cell transcriptome. *Proc Natl Acad Sci U S A* 101:10143-8, 2004
8. Sridhar S, Schembri F, Zeskind J, et al: Smoking-induced gene expression changes in the bronchial airway are reflected in nasal and buccal epithelium. *BMC Genomics* 9:259, 2008
9. Kikuchi T, Hassanein M, Amann JM, et al: In-depth Proteomic Analysis of Nonsmall Cell Lung Cancer to Discover Molecular Targets and Candidate Biomarkers. *Mol Cell Proteomics* 11:916-32, 2012
10. Zhang H, Liu Q, Zimmerman LJ, et al: Methods for peptide and protein quantitation by liquid chromatography-multiple reaction monitoring mass spectrometry. *Mol Cell Proteomics* 10:M110 006593, 2011